POLITECNICO DI TORINO Repository ISTITUZIONALE

Exploring the effect of utilising organic acid solutions in ultrasound-assisted extraction of pectin from apple pomace, and its potential for biomedical purposes

Original

Exploring the effect of utilising organic acid solutions in ultrasound-assisted extraction of pectin from apple pomace, and its potential for biomedical purposes / Girón-Hernández, Joel; Pazmino, Michelle; Barrios-Rodríguez, Yeison Fernando; Turo, Chiara Tonda; Wills, Corinne; Cucinotta, Fabio; Benlloch-Tinoco, Maria; Gentile, Piergiorgio. - In: HELIYON. - ISSN 2405-8440. - 9:7(2023), p. e17736. [10.1016/j.heliyon.2023.e17736]

Availability: This version is available at: 11583/2982996 since: 2023-10-13T14:07:31Z

Publisher: CELL PRESS

Published DOI:10.1016/j.heliyon.2023.e17736

Terms of use:

This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

Publisher copyright ASA postprint versione editoriale con licenza CC BY/Version of Record with CC BY License

Copyright 2023 Author(s). This article is distributed under a Creative Commons Attribution (CC BY) License.

(Article begins on next page)

1	
Τ.	

The influence of organic acids coupled with ultrasound on pectin extracted from apple pomace for biomedical applications

3	Joel Girón-Hernández ^{1*} , Michelle Pazmino ¹ , Yeison Fernando Barrios-Rodríguez ^{2,3} , Chiara Tonda
4	Turo ⁴ , Corinne Wills ⁵ , Fabio Cucinotta ⁵ , Maria Benlloch-Tinoco ¹ , Piergiorgio Gentile ^{6*}
5	¹ Department of Applied Sciences, Faculty of Health and Life Sciences, Northumbria University,
6	NE1 8ST Newcastle upon Tyne, United Kingdom
7	² Department of Food Technology, Universitat Politècnica de València, Camino de Vera s/n, 46021
8	Valencia, Spain
9	³ Centro Surcolombiano de Investigación en Café (CESURCAFÉ), Universidad Surcolombiana,
10	410007 Neiva, Colombia

⁴ Department of Mechanical and Aerospace Engineering, Politecnico di Torino, 10129 Turin, Italy

⁵ School of Natural and Environmental Sciences, Newcastle University, NE1 7RU Newcastle upon

13 Tyne, United Kingdom

⁶ School of Engineering, Newcastle University, NE1 7RU Newcastle upon Tyne, United Kingdom

15 Corresponding authors: *Joel Girón-Hernández joel.l.g.hernandez@northumbria.ac.uk and

- 16 Piergiorgio Gentile <u>piergiorgio.gentile@ncl.ac.uk</u>
- 17 Abstract

18 Biomass resulting from food production represents valuable material to recover different

19 biomolecules. In our study, we used apple pomace to obtain pectin, which is traditionally extracted

- 20 using mineral acids. Our hypothesis consisted of carrying out extractions with organic acids,
- assisted by ultrasound, considering variations in the factors of time, temperature, and type of acid.
- 22 The analytical determinations of galacturonic acid content, methoxylation and esterification degree,
- 23 ζ-potential and extraction yield were used as pectin quality indicators. Treatments with better

performance were assessed biologically in vitro for their potential to be used in biomedical 24 applications. Overall, the extracted pectin presented a galacturonic acid content, methoxylation and 25 esterification degree ranged from 19.7-67%, 26.8-41.4% and 58-65.2% respectively, and were 26 27 negatively charged (-24.1 to -13.2 mV). It was found that factors of time and temperature greatly influenced the response variables excepting the esterification degree, while the acid type influenced 28 the ζ-potential, methoxylation and esterification degrees. Additionally, it was seen that the longer 29 extraction time (50 mins) and the higher the temperature (50 °C) exhibited the better extraction 30 yield (~10.9%). Finally, the selected pectin showed high cytocompatibility up to 500 µg/mL of 31 concentration when seeded with Neonatal Normal Human Dermal Fibroblasts. 32

Keywords: Apple pomace, Pectin, Ultrasound-assisted extraction, Principal Component Analysis,
Biomaterials.

36 1. Introduction

Valorisation of agro-industrial biowaste is a smart strategy that must be achieved through efficient 37 and reproducible approaches, valuing green chemistry principles. Particularly, the extraction and 38 purification of bioactive compounds can impact socio-environmental demands or economic 39 challenges [1]. Although apple crop in 2022 was affected by weather conditions in Asia, around 79 40 million tonnes of this fruit was produced worldwide [2]; in this scenario, value-added apple 41 products such as juice, cider, jam and dried, account for 25-30% of the above volume, leading to a 42 pomace biowaste mass that can reach up to 25% of the fresh fruit weight [3]. Particularly, apple 43 pomace is a valuable material for extracting high attractive biomolecules like carbohydrates, 44 polyphenols and triterpenes [4]. Pectin (PEC) is an interesting molecule present in vegetable cell 45 walls and could be recovered from apple pomace and other vegetable biomass sources [5], it is a 46 carbohydrate polymer with plenty of applications in the food sector. Traditionally, PEC has been 47 used as gelling or thickening agent, this stabiliser property is complemented by the attractive 48 utilisation of pectin as a fat replacer and health-promoting functional ingredient [6]. Alternative 49 emerging applications include the use in the biomedical and pharmaceutical industries. Pectin, due 50 to its simple and cytocompatible gelling mechanism, has been recently exploited for different 51 biomedical applications including drug delivery, gene delivery, wound healing, and tissue 52 engineering [7]. Indeed, natural biopolymers are at the centre of materials development for 53 biomedical and biotechnological applications based on their low-toxicity, biodegradability and 54 biofunctional key features [8]. 55

Current literature reports several works focused on PEC extraction from apple pomace; on a commercial scale, diverse conditions are carried out for its purpose. However, PEC is generally extracted trough water-mineral acidic solution (sulfuric, nitric, phosphoric, hydrochloric) at a pH around 1.5, where the biomass is heated at temperatures ~80 °C, followed by an ethanol precipitation at different concentrations, from 70% to absolute [9, 10]. Above-mentioned parameters can lead easily to equipment corrosion and environmental pollution derived from the

acidic wastewater disposal [11]. Therefore, experimental studies with apple pomace or peel, have 62 been conducted for exploring alternatives procedures to make PEC extraction process more 63 sustainable and to enhance its recovery. In this sense, methodologies such as: organic acid 64 65 extraction, application of eutectic solvents, sequential extraction, enzymatic extraction, assisting extraction with microwaves, radio frequency, ultrasounds or the combination of this methodologies 66 have been proposed. Indeed, Cho et al. [12] have compared different acidic extractions, by using 67 mineral and organic acids; they found that similar amounts of pectin were extracted (~6.6%) with 68 1M organic acids (tartaric, malic, citric) with an esterification degree ranged from 54 to 64.8% 69 compared with conventional extraction (~6.4%) by using HCl. Furthermore, a two-step slight acidic 70 process using H₂SO₄ (pH 2.4) under hot stirring (100 °C) was conducted for 110 mins, leading to a 71 PEC extraction yield of ~15%, the debris remained from the process, were used to extract cellulose-72 rich substances and monosaccharides, obtaining a recovery rate of 38-49% respectively [13]; this 73 experiment represents a complete valorisation example of apple pomace; however, PEC extraction 74 was carried out using conventional methods. Other alternative involving eutectic pre-treatments can 75 be considered, where glycerol and lactic acid have been mixed either with choline chloride (pH 1-76 6.5), potassium carbonate (pH 12-14), urea or oxalic acid, leading to a final yield of extracted PEC 77 in the range of 6-8.5% with a methoxylation degree ranged from 54 to 79%, and with an overall 78 recovery of neutral sugars between 76-87% [14] [11]. Nevertheless, this sequential extraction lasted 79 more than 48 h and PEC extraction yield was not significantly high compared with findings of other 80 authors that explored different methodologies; for example, mediating the extraction process with 81 enzymes, it was obtained ~7% of extraction yield, and the result did not present a much better 82 performance when assisted with ultrasound (\sim 8%). Although, in the same experiment when 83 84 changing the conditions to citric acid as extractant solution at pH 2.2 and microwave assisted at pH 1.8, PEC recovery was improved up to ~23% for both conditions [10]. Recently, Zheng et al [15] 85 combined the use of citric acid solutions at a pH ranged from 1.5 to 2.5 with microwave (MWAE) 86 and radio frequency (RFAE) assisted extractions, reaching temperatures between 80-90 °C for 20 87

88	minutes. Both MWAE and RFAE procedures helped to get an extraction yield of ~11%, that
89	resulted in a higher performance compared with citric acid extraction at pH 2.2 as control (~7.5%
90	PEC recovery). Furthermore, following RFAE method, higher content of galacturonic acid content
91	(~63%) and esterification degree (~66%) were reported compared with MWAE and citric acid
92	control (~41 and ~51% for the galacturonic acid, and ~54 and ~59% for the esterification degree
93	respectively). Thus, microwaved and radiofrequency techniques can substantially reduce duration of
94	the extraction; however, their execution could result demanding because batch processing is
95	required [16]; additionally, microwaves generate uneven heating due to high temperature, that
96	might cause degradation of the components in the outermost areas of the mass volume being
97	extracted [17].
98	Finally, Dranca et al [18] proposed the use of citric acid solutions, assisted with ultrasound, up to 30
99	minutes of extraction process. They found out that at maximum ultrasound amplitude and lower pH,
100	PEC extraction yield and degree of esterification presented the higher values (9.1% and 88.5%
101	respectively). Compared to the MWAE and RFAE, the ultrasound assisted procedure allows to
102	preserve the physico-chemical structure of the extracted pectin [19].
103	Therefore, in our study we conducted a series of PEC extractions from apple pomace, ultrasound
104	assisted, by comparing two different organic acids solutions (acetic and citric), aiming at evaluating
105	the impact of time and temperature on PEC quality (galacturonic acid content, methoxylation and
106	esterification degree and electrostatic charge) and extraction yield. Additionally, the obtained pectin
107	with higher galacturonic acid content and extraction yield were assessed biologically in vitro by
108	using Neonatal Normal Human Dermal Fibroblasts (NHDF) for their potential to be used in
109	biomedical applications.

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4393532

110 **2.** Materials and methods

111 2.1 Materials and chemicals

112 Glacial acetic acid (ACS reagent, \geq 99.7%), citric acid (ACS reagent, \geq 99.5%), hydrochloric acid

- 113 (ACS reagent, 37%), ethanol 96%, sodium chloride (ACS reagent, ≥99.0%), phenol red (indicator
- 114 ACS), sodium hydroxide (reagent grade, ≥98%), m-hydroxydiphenyl, D-(+)-Galacturonic acid
- 115 monohydrate (analytical standard), sodium tetraborate, sulphuric acid (ACS reagent, 95.0-98.0%)
- and all other chemicals were purchased from Sigma-Aldrich, UK. Deionised water was obtained
- 117 throughout Milli-Q[®] Water Purification System (IQ 7005, Merk, UK).

118 **2.2** Apple biowaste processing and preparation

Apples (*Malus domestica* Bork) var. Royal Gala, from different origins (France, UK, South Africa, Chile), were purchased in a local supermarket. Subsequently, samples were visually verified to remove any damaged areas and hand-washed with tap water. Then, they were cut and ground using a fruit juicer (Cookworks, Argos, UK). The resulting pulp was passed through the juicer 3 more times to maximise the water removal and get smaller solid particles. Apple pomace yield in relation to whole apple and moisture content of apple pomace were determined by using the AOAC method [20], while the soluble solids from the extracted juice were measured by using a digital

126 refractometer (RS PRO, UK).

127 Wet apple pomace was dried at 68°C in a vacuum oven (SVAC1-2, SHEL LAB, UK) for 24 h

128 before milling with an electric grinder (Blender LB20E, Waring Commercial, US) into powder and

then, stored in grip seal bags in desiccator until further use.

130 **2.3 Experimental design of the pectin extraction from the apple pomace**

131 Extraction of pectin from apple pomace was carried out using a combination of variables including

132 acidic solution from acetic acid (AA) or citric acid (CA), sonication time (25 or 50 min) by using an

133 ultrasound water bath and temperature at 40 and 80 °C. The processing parameters were selected

based on the most reported values in literature for successfully extracting pectin from other foodwaste biomasses [21-23].

136 Ultrasound assisted extraction was performed by mixing 15 g of apple pomace powder with 300 mL

137 (to reach a ratio of 1g/20 mL) of distilled water in which citric acid or acetic acid was added to

reach a pH value of 1.5 by titration with 1M HCl. The ultrasound water bath (USC 300T, VWR,

139 UK) was set at 45 kHz, 80 W, and 100% amplitude. After sonication the mixture was centrifuged at

140 4400 rpm for 20 mins (SORVALL ST 8R, Thermo-Fisher, UK), and the supernatant was collected,

141 filtered using a nylon mesh, and transferred to standard glass flasks. Equal amount of ethanol was

added to the supernatant and the resulting solution was kept for 24 h at 4-6 °C. Then, the

143 precipitated pectin was centrifuged at 4400 rpm for 10 min and consecutively washed with ethanol

144 while filtering through nylon mesh. The resulting pectin was dried at 45 °C on a heated incubator

145 (MIR-162, Panasonic, Japan) until constant weight and kept and stored in grip seal bags in

146 desiccator until further use.

147 The yield of the extracted pectin was calculated with the following formula (Eq.1):

148 Pectin yield (%) = $\frac{dried \ pectin \ weight}{dried \ apple \ pomace \ weight} \times 100$ Eq.1

149 2.4 Characterisation of the extracted pectin

2.4.1 Determination of the anhydrouronic acid contents and the degree of methoxylation and esterification

152 The degree of methoxylation (DM) and anhydrouronic acid (AUA) contents and degree of

esterification (DE) in pectin samples were analysed by conventional methods [21, 24]. To 50 mg of

- 154 pectin, 500 μL of ethanol, 10 mL of distilled water, 0.10 g NaCl and one drop of phenol red
- indicator were added. The solution was stirred for 15 min to dissolve all of the components, and
- then titrated with 0.1 M NaOH until the colour changed (Titration A). Subsequently, 2.5 mL of 0.25
- 157 M NaOH was added to the mixture and allowed to stand for 30 mins at room temperature. Finally,
- 158 2.5 mL of 0.25 M HCl was added, and the mixture was titrated again with 0.1 M NaOH until the

159 colour turned red (Titration B). The degree of methoxylation was calculated by using the following

160equation (Eq.2):161
$$DM(\%) = \frac{meq Titration B \times 31 \times 100}{weight of sample (mg)}$$
Eq.2162Where meq Titration B are the milliequivalents of NaOH used for the Titration B, and 31 is the163molecular weight of the methoxyl group.

164 The anhydrouronic acid content was calculated according to the equation 3 (Eq.3):

165
$$AUA(\%) = \frac{176 \times 100}{z}$$
 Eq.3

166 Where 176 is the molecular weight of AUA and

167
$$z = \frac{weight of sample (mg)}{meq Titration A + meq Titration B}$$
 Eq.4

168 Finally, the degree of esterification of the extracted pectin was calculated by:

169
$$DE(\%) = \frac{176 \times DM\% \times 100}{31 \times AUA\%}$$
 Eq.5

170 **2.4.2** Galacturonic acid content analysis

A colorimetric method based on the m-hydroxydiphenyl reagent was used to measure the total 171 galacturonic acid (GA) content of the extracted pectin following the protocol proposed by 172 Gharibzahedi et al. [25]. Briefly, 500 µL of pectin solution (concentration of 200 µg/mL) was 173 174 poured into a glass tube vial, and then 3 mL of sulfuric acid/sodium tetraborate was added and immediately cooled in a bath containing cold water. A continuous operation including shaking the 175 tubes for 30 s with a vortex mixer (VORTEX 3, IKA, Germany), heating in a water bath (GLS 176 Aqua 12 Plus, Grant, UK) at 100 °C for 5 mins and cooling in ice water was performed. Then, 100 177 µL of m-hydroxydiphenyl (0.15% in 0.5% NaOH) were added to the vial and kept under shaking 178 for 5 minutes (SSM1, Stuart, UK). Finally, the absorbance of the resulting solutions was read at 179 525 nm using a multiplate reader (FLUOstar Omega, BMG Labtech, Germany). For the preparation 180 of the calibration curve, solutions of galacturonic acid (between $1-200 \text{ mg} \cdot \text{mL}^{-1}$) were used. 181

182 2.4.3 NMR measurement

183 The extracted pectin samples were analysed by NMR spectroscopy. Saturated samples were

prepared in 0.7 mL D₂O with TMSP-d4 [3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid]

185 (Sigma-Aldrich, UK) added as an internal reference (0.0 ppm). The ¹H NMR spectra were obtained

at 80 °C on a Bruker Avance III HD 700 MHz NMR spectrometer using a Prodigy TCI cryoprobe.

187 Each spectrum was acquired with 16 scans and 32 K datapoints (transformed to 128 K). Baseline

188 corrections were applied before integration.

189 2.4.4 Molecular weight determination

190 The molecular weight of the extracted pectin was assessed by size-exclusion chromatography (SEC;

191 1260 Infinity GPC/SEC System, Agilent), equipped with a PL aquagel-OH MIXED-H 8 μm

192 column. The samples were dissolved overnight at 2 mg/mL concentration in a recommended buffer

193 $(0.2 \text{ M NaNO}_3 + 0.01 \text{ M NaH}_2\text{PO}_4 \text{ at pH 7})$, and, then, filtered through a 0.45 μ m membrane (Titan

194 3, PTFE, ThermoScientific, UK) prior to injection (20 μ l). The column set was calibrated with

195 narrow pullulan standards and, thus, all molecular weight values were determined.

2.4.5 Fourier transform infrared spectroscopy (FTIR-ATR)

197 FTIR-ATR spectroscopy analysis was performed on the extracted pectin. The infrared spectra were 198 obtained with a spectrophotometer Spectrum one equipped with UATR accessory. The readings 199 were taken in the wavelength range of 4000–550 cm⁻¹, for each of the eight independent samples of 200 each combination: acidic solution x sonication time x temperature, at least five consecutive readings 201 were taken from pectin flakes. The average value was considered as representative for each sample.

202 **2.4.6** ζ -potential measurement

The ζ-potentials of pectin solutions (1:1 mg mL⁻¹) were measured by laser Doppler electrophoresis
(Zetasizer Nano, Malvern instrument, US). Three sets of at least 10 measurements were averaged to

205 get the final ζ -potential value for each PEC solutions.

206 2.4.7 Rheological analysis

PEC solutions were solubilised in deionized water at 2% (w/w) under stirring at 25 °C for 16 h, then 207 solutions were allowed to rest overnight at 4°C prior to the rheological experiments. The tests were 208 performed by using a stress-controlled rheometer (MCR302, AntonPaar GmbH, Graz, Austria) 209 equipped with 25 mm parallel plate geometry. For each test, each pectin solution was poured on 210 lower plate at 25°C. De-hydratation was prevented by a water trap while temperature control was 211 guaranteed with a Peltier system. The shear strain amplitude on each pectin solution was measured 212 by the shear strain test at 25°C (rotational oscillation 1 Hz, strain from 0.01% to 500%), while the 213 frequency sweep test was performed using angular frequencies (ω) from 100 to 0.1 rad/s and a 214 strain value within the linear viscoelastic region of 1%. Furthermore, the solution viscosity was 215 determined using a shear rate from 0.1 to 100 1/s and a strain of 1%. Rheological tests were 216 performed in triplicate. 217

218 **2.4.8** Pectin as biomaterials: *in vitro* cell tests

219 *2.4.8.1 Cell culture and seeding*

220 Neonatal Normal Human Dermal Fibroblasts (NHDF) were purchased from Lonza Biosciences (Switzerland) and cultured as recommended by the seller. Briefly, fibroblasts were grown at 37 °C, 221 5% CO₂, in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal 222 bovine serum (FBS), 2 mM L-glutamine and a 1% antibiotic mixture containing penicillin and 223 streptomycin (100 U mL⁻¹). To perform biocompatibility assays, PEC solutions at different 224 concentrations (10, 25, 50, 100, 250, 500 and 1000 µg/mL) were prepared by dissolving the pectin 225 powders in DMEM and then sterilised by filtration through a 0.22mmMillex GP PES membrane 226 syringe-driven filter unit (Millipore, SLS, UK) using 5 ml plastic syringes. Suspensions of 8 x 10⁴ 227 cells and 10 x 10⁴ cells in DMEM were seeded on each well of a 96 and 48-multiwell plates 228 respectively, with the different diluted PEC solutions, and then incubated with at 37 °C, 5% CO₂ for 229 the necessary biological tests. 230

231 2.4.8.2 Cytocompatibility studies

Cell viability was assessed with the live/dead staining (LIVE/DEAD® Cell Imaging Kit, Life
Technologies, Thermo Scientific, US) at 24 h in 48-multiwell plates. According to the
manufacturer's protocol, membranes were washed with phosphate buffered saline (PBS, SigmaAldrich, UK) and stained with 150 µl solution of 4 µM Ethidium homodimer-1 and 2 µM calcein in
PBS. After 30 min of incubation at room temperature, cells were imaged with a EVOS M5000
fluorescence microscope to detect calcein (ex/em 488 nm/515 nm) and Ethidium homodimer-1
(ex/em 570 nm/602 nm), respectively.

Furthermore, at the same time point, Presto Blue assay was exploited to test the metabolic activity
of cells seeded with the different diluted PEC solutions in 96-multiwell plates. A Filter-based
FLUOstar® Omega multi-mode reader (FLUOstar® Omega, Germany) was used to measure the
fluorescence (560 nm excitation and 590 nm emission) after 1.30 h of incubation with a 10%
aliquot of Presto Blue (Thermo Scientific, USA). Results were expressed as mean ± standard

244 deviation.

Finally, the cell morphology was observed by nucleus and cytoskeleton staining after 48 hours of
cell seeding. Briefly, cells were fixed with 4% paraformaldehyde solution for 15 min, followed by
three washing steps with PBS. Cells were then permeabilised using 0.1% v/v Tween20® in PBS for
5 min. Rhodamine-phalloidin was prepared using 1:100 dilution of phalloidin-

tetramethylrhodamine B isothiocyanate (Sigma Aldrich, P1951) in 1% v/v Tween20® in PBS for

250 30 min, and then washed three times with PBS. One drop of DAPI (VECTASHIELD®) antifade

mounting media was added to each sample, then covered with a glass slide and imaged using a

EVOS M5000 fluorescence microscope.

253 2.4.9 Statistical analysis

254 The analytical determination results were processed by one-way ANOVA, with mean separation by

255 Tukey's test at 95% confidence level. A multifactor ANOVA was performed on the extraction

- 256 parameters: Acid type (A), Extraction time (Et), Temperature (Tp) and their interactions, to evaluate
- their effects on the analytical determinations performed on the extracted pectin. The infrared

- 258 information was analysed by Principal Component Analysis (PCA) to group the different
- extractions. The spectra were pre-processed to compensate and remove the bias linked to the
- 260 experimental assessment by baseline correction (MicroLab Expert, FTIR Software, Agilent, US).
- 261 Subsequently, different methods such as standard normal variance (SNV), multiplicative dispersion
- correction (MSC), and first and second derivatives were evaluated on the range of 1800 650 cm⁻¹
- 263 of the spectra (known as fingerprint) that provided key information to differentiate samples from
- different treatments [26]. Data processing was performed using Statgraphics Centurion 19
- 265 (Statpoint Technologies, Inc., USA) and R statistical software (version 3.6.3, R statistics, US).

266 **3. Results**

268

267 **3.1 Analytical determination**

soluble solids and moisture content, presenting 12.41±0.62° Brix, and after removing the water 269 from the pomace, dry matter represented $19.63\pm0.43\%$ apple pomace (dry base). 270 The effect of ultrasound-assisted extraction with the combination of different processing parameters 271 (acid type, temperature, and time of extraction) on the analytical properties of the extracted pectin 272 have been investigated in this work. These results are summarised in Table 1 and elaborated by the 273 multifactor ANOVA to investigate if these variables were statistically significant or not (Table 2). 274 The yield of the pectin obtained from the different extractions ranged from 1 to 12%, depending on 275 the type of acid, time, and temperature of extraction. Particularly, it can be observed that the yield 276 increased with increasing time and temperature. As example, for the citric acid, the yield increased 277 from 3.1±0.7% at 40 °C for 25 min to 11.8±1.5% at 80 °C for 50 min of extraction. According to 278 the F-ratio reported in **Table 2**, the temperature of extraction (T_p) presented the highest influence 279 (47.33 ***) followed by the extraction time (E_t , 17.18 **) while the acid type (A) presented a 280 statistical effect only in interaction with the other 2 processing factors ($A \times E_t \times T_p$, 11.49 **). Thus, 281 temperature was a crucial parameter, because its increase allowed the increase of pectin solubility, 282 resulting in a higher yield. The behavior was described in literature in different works reporting 283 extraction of pectin from different biomass [27, 28]. 284 The same substantial influence of the time and temperature of extraction was observed for the 285 content of galacturonic acid (E_t , 239.36*** and T_p , 792.12***). GalA is the most prevailing 286 building block of pectin, which makes its determination a very important step in the analysis of 287 pectin's chemical structure [29]. The range of the analysed galacturonic acid was between ~20-50% 288 with the highest content found in the pectin extracted with acetic acid at 80 °C for 50 minutes. 289

In this work, Royal gala apples have been bought from a local store and they were characterised by

290 Commercial apple pectin purchased from Sigma-Aldrich was used as control and it was found to be

characterised by 67% GalA within the range reported into the specification sheet of the supplier.

Furthermore, the degree of esterification (DE) is another parameter that affects pectin quality and applications. Indeed, according to the extraction conditions, different proportions of the acid groups of the GalA units are esterified and this is knows as DE [30]. Moreover, GalA units can be partly methoxylated, where the backbone presents methyl ester forms (-COOCH₃), and this can be

calculated as degree of methoxylation (DM) [31].

In our work, all the extraction conditions led to a DE ranging from 58 to 65%. In contrast with the 297 other analytical determinations, the use of the different acid type influenced the DE (5.46 *) where 298 the citric acid extractions provided the highest values (at 80 °C for 25 minutes, 63.0±5.6% for CA 299 respect with 58.0±0.3% for AA). As shown in Table 1, both acids presented a similar DE to that of 300 commercial SIG-APP (58.9±2.4%). Numerous researchers described that the pectin solubilisation 301 into the solvent happened due to the breakage of the plant cell wall under the influence of the 302 ultrasound [32, 33]. Particularly, ultrasound is a green method that rises the selectivity, decreases 303 reaction time, and encourages macro- and micro- mixing via acoustic cavitation, creating 304 cavities/bubbles. After collapse, these can release huge amounts of energy that is made available to 305 break the structure where pectin is contained [34]. As demonstrated by Zhang et al. [35] high 306 intensity ultrasound (up to 300 W cm⁻²) can increase the DE >70%. They reported a similar value of 307 DE close to 60% when using a lower ultrasound power (~60W cm⁻²) at 20 °C for 30 minutes. 308 Then, according to the DM, pectin can be categorised as high methoxy pectin (DM > 50 %) and low 309 methoxy (DM \leq 50 %) [36]. Table 1 shows that the DM values of all the apple pectin were in the 310 ranges of ~27-41%; thus, our pectin can be classified as low methoxyl. Moreover, the pectin with 311 the highest DM was obtained with the citric acid by comparing the same extraction conditions (time 312 and temperature) of the acetic acid. This trend was confirmed by the F-ratio (21.5**). These 313 314 considerations on the DM are important for selecting the use of the pectin in biomedical application as bioink and hydrogel for tissue engineering and regenerative medicine. Particularly, low or high 315 DM require different conditions for crosslinking pectin. Pectin with low DM is characterised by 316 high number of free carboxyl groups with high cation-binding ability. The binding of divalent 317

318	cations e.g. Ca ²⁺ , Mg ²⁺ produces junction zones between two polyguluronate chain dimers. These
319	segments present an "egg-box" structure, where the binding of the cation to the carboxyl groups of
320	two opposite pectin chains was stabilised by van der Waals interactions and hydrogen bonds [37].
321	Thus, our extracted pectin in all the conditions can be suitable to manufacture bioprinted constructs
322	or <i>in situ</i> gelling systems. Indeed, the ζ -potential values ranging from -13 to -24 mV confirmed the
323	presence of a high number of free COOH groups, fundamental for the further ionotropic gelation
324	with divalent ions. Furthermore, this negative charge of the extracted pectin can allow to use it as
325	polyelectrolyte (specifically as polyanion) for the surface functionalisation of medical devices by
326	technique of Layer-by-Layer (LbL) assembly. LbL is an environmental-friendly technique that
327	allows to create a multilayered coating at the nanoscale, exploiting the electrostatic interaction of
328	polyelectrolytes, for modifying the surface topography and/or entrapping biomolecules/drugs to
329	impart specific biological activities [38, 39].

Table 1. Yield, galacturonic acid content, methoxylation and esterification degree, and ζ -potential of the extracted pectin samples from apple pomace obtained by conventional acidic extraction at pH= 1.5 with different temperatures and times. The values are shown as average ± SD.

Code	Acid	Temp.	Time	Yield	GalA	DM	DE	ζ-potential
	1 ICIU	(°)	(min)	(%)	(%)	(%)	(%)	(mV)
CA40-25	CA	40	25	3.1±0.7	27.1±4.8	41.4±2.0	59.1±1.3	-22.9±1.1
CA80-25	CA	80	25	7.1±1.9	31.0±3.9	37.5±2.1	63.0±5.6	-13.2±0.7
AA40-25	AA	40	25	1.2±0.3	19.7±0.5	33.8±3.2	58.1±1.9	-22.7±2.8
AA80-25	AA	80	25	10.8±2.9	43.9±0.7	27.5±0.8	58.0±0.3	-16.1±0.4
CA40-50	CA	40	50	5.0±0.3	36.9±2.0	36.6±3.1	65.2±4.4	-13.5±1.2
CA80-50	CA	80	50	11.8±1.5	43.7±2.6	33.2±1.4	61.4±1.4	-15.8±0.2
AA40-50	AA	40	50	8.6±2.3	24.4±1.4	32.0±4.4	61.2±5.2	-20.9 ± 0.8
AA80-50	AA	80	50	10.1±2.4	49.2±2.4	26.8±1.7	58.2±3.4	-18.6±0.5
SIG-APP		-		-	67.0±2.6	31.9±1.3	58.9±2.4	-24.1±1.1

Table 2. F-ratio values and significance levels obtained in multifactor ANOVA for the physico-

chemical parameters according to the factors: Acid type (A), Extraction time (E_t) , Temperature (T_p) and their interactions.

Α	Et	T _p	A x E _t	A x T _p	$\mathbf{E}_{\mathbf{t}} \mathbf{x} \mathbf{T}_{\mathbf{p}}$	A x E _t x T _p

Yield (%)	1.37 ^{NS}	17.18**	47.33***	$0.0^{\rm NS}$	0.01 ^{NS}	2.72 ^{NS}	11.49**
GalA (%)	0.72 ^{NS}	239.36***	792.12***	35.67***	320.16***	2.95 ^{NS}	1.44 ^{NS}
DM (%)	21.5**	4.57*	10.11*	1.21 ^{NS}	0.73 ^{NS}	0.24 ^{NS}	0.01 ^{NS}
DE (%)	5.46*	1.72 ^{NS}	0.19 ^{NS}	0.06^{NS}	$0.24^{\rm NS}$	3.18 ^{NS}	$0.74^{\rm NS}$
ζ-potential (mV)	57.23***	12.49**	91.87***	18.94**	0.72 ^{NS}	88.63***	19.79**

336 ^{NS}, not significant. *p<0.05, **p<0.01, ***p<0.001.

¹H NMR spectra of the extracted and commercial apple pectin were compared. All the spectra were 337 338 characterised by a broad signal chain (i.e. CH₃ and CH₂ groups) ranging from 0 to 2.5 ppm [40] (Figure S1). Particularly, signals at 2.11 and 1.91 ppm are from the -COCH₃ groups located at 3-O-339 and 2-O-galacturonic acid. Then, signals at 1.30 ppm and 1.27 ppm are from the CH₃ group of L-340 rhamonose. The peak at 3.92 ppm is derived from the CH₃ group that is associated with the 341 carboxyl groups of GalA. The remaining pectin signals are assigned to the 5 protons found in GalA 342 (H₁, 4.97 ppm; H₂, 3.73 ppm; H₃, 3.97 ppm; H₄, 4.16 ppm, and H₅, 4.70 ppm) (labelled in blue in 343 Figure 1 and reported in Table 3). Furthermore, signals at 5.13 ppm and 4.92 ppm located in the 344 345 anomeric region are assigned to H₁ Rha and H₁ Gal, respectively. Furthermore, the extracted pectin 346 showed differences compared with the control SIG-APP. Indeed, the acetyl groups of GalA acid and methyl groups of Rha were not visible in the ¹H NMR spectrum at range 2.5-1 ppm (Figure 347 S1). Also, the extracted pectin showed a visible increase in the intensities of the peaks at 4.92 ppm 348 of the H_1 Gal, that could overlap the peek at 4.97 ppm of H_1 GalA, and at 4.70 ppm of the H_5 GalA. 349 However, all the other protons, characterising the GalA [41], were less intense or not detected. 350



Figure 1. ¹H NMR spectrum of pectin from apple pomace extracted by using acetic acid at 80°C for 25 minutes ultrasound-assisted.

Table 3. ¹H NMR chemical shifts of pectin from apple pomace extracted by using acetic acid at
 80°C for 25 minutes ultrasound-assisted.

	H ₁	H ₂	H ₃	H_4	H_5	H ₆
D-galacturonic acid	4.97	3.73	3.97	4.16	4.70	n.d.
L-rhamonose	5.13	4.31	3.88	3.67	4.02	n.d.
D-galactose	4.92	3.64	4.10	3.87	4.41	3.66

n.d.= not detected.

351

357 FTIR-ATR pectin spectra obtained after acidic extraction for all the different treatments are

illustrated in Figure 2. The main absorption peaks recorded around 3600-3000 cm⁻¹ were caused by

- 359 O-H stretching, while characteristic absorption peak of pectin-reproduced polysaccharides due to C-
- H stretching of CH_2 groups was observed between 3000-2800 cm⁻¹ [18, 24]. Stretching vibration
- 361 (C=O) of methyl-esterified and carboxylate ions (free carboxyl groups) of pectin resulted in the
- bands at 1743 cm⁻¹ and 1631 cm⁻¹, respectively [42]. The tendency of increasing intensities and

band area of esterified carboxyl groups may indicate an increase in degree of esterification [43]. 363 Certainly, esterified carboxyl groups exhibit an increasing trend in their intensities and band areas, 364 as esterification degree value increases [44]. Also, the higher absorbance for esterified carboxylic 365 366 groups, compared to free carboxylic groups, would indicate a higher degree of esterification [45]. Bands related to the stretching of the C-O bond were observed between 1300 and 1000 cm⁻¹ [24], 367 while the absorption band at 1225 cm⁻¹ was due to the cyclic C-C bond in the ring structure of 368 pectin. Finally, the region between 1120-990 cm⁻¹ has been reported for the spectral identification 369 of galacturonic acid in peptide polysaccharides [46]. 370



371

Figure 2. ATR-FTIR spectra with baseline correction of the apple pectin samples obtained in the 372 mid-infrared 4000-650 cm⁻¹ range after acidic extraction with citric (A) and acetic acid (B). 373 374 Furthermore, some more considerations can be done on mid-infrared (MIR) spectra where the wavenumber range of 4000-650 cm⁻¹ can be classified into two different regions: functional group 375 376 (4000-1500 cm⁻¹) and fingerprint region (1500-650 cm⁻¹). In both regions, changes in absorbance values are observed due to the different treatments (Figure 2). However, differences in the 377 fingerprint region are more evident in peaks of interest such as those associated with the degree of 378 esterification (743 cm⁻¹ and 1631 cm⁻¹), gallic acid (1120-990 cm⁻¹) and pectin structure cycle 379 380 (1300-1000 cm⁻¹). Therefore, the chemometric analysis of the spectrum was performed within information in the region of 1800-650 cm⁻¹wavenumbers. 381 Following the baseline correction, an exploratory PCA analysis was performed with the information 382 of the region between 1800-650 cm⁻¹ for 25-minute treatments, using different processing 383

techniques such as SNV, MSC, and first and second derivatives. The best clustering results were
evidenced with MSC, which are illustrated in Figure 3. As can be seen, the first two components
explain almost all the variability of the MIR information (77.24%) (Figure 3A). The first principal
component (PC1) provides the main contribution (50.78%), while the second (PC2) explains
26.46%.



389

390 Figure 3. (A) PCA of the processed infrared signal spectra of extracted pectin (25 min) with baseline correction + MSC normalisation; (B) Pectin apple spectrum and loadings for PC1 and PC2. 391 The scatter plot shows three different groups according to the treatment applied: i) AA40, ii) AA80 392 and iii) CA40 and CA80. This shows a clear effect on pectin composition when temperature is 393 varied from 40 to 80 °C in the acetic acid extraction, while this effect is not observed with citric 394 395 acid. Thus, FTIR analyses confirmed the influence of temperature during the extraction on the pectin structure and the content of GAs, which were lower in AA40 than those obtained in AA80 396 (Table 1), which could explain the differences evidenced by the analysis of the IR spectra. 397 The loadings plot for the first two components indicates that the region between 1800 to 1700 cm⁻¹ 398 and 1420 to 1180 cm⁻¹ are strongly associated with the samples in grouped PC1 positive region, 399 where CA40 and CA80 samples were located. As discussed above, these regions are associated with 400 401 the degree of esterification and C-O stretching, respectively. This is consistent with the significant higher degree of esterification for the samples extracted with citric acid (Table 1 and 2) An 402 important contribution from the region between 1200-900 cm⁻¹, it is also evident in the negative part 403 of PC1 where most of the AA40 samples were observed. This zone could be influenced by the 404





Figure 4. (A) PCA of all processed infrared signal spectra of extracted pectin (25 and 50 min) with
baseline correction + MSC normalisation; (B) Pectin apple spectrum and loadings for PC1 and PC2.

Finally, the molecular weight is a key-parameter for evaluating the relationship between

427 polysaccharide structure and function [47], where its value is associated with the pectin gelling

428 properties, fundamental for being considered suitable for the manufacturing of hydrogels in tissue

engineering [48] The M_W of the extracted pectin samples ranged from 1.11 to 1.15 x 10⁵ Da. The

430 commercial pectin had similar value $(1.13 \times 10^5 \text{ Da})$ in accordance with the literature [49].

431 Therefore, no differences have been noticed among all the extracted pectin samples.

432 **3.2 Rheological analysis**

Rheological analysis measurements reported a different behaviour for extracted pectin solutions 433 from the apple pomace compared to commercial pectin from Sigma-Aldrich (SIG-APP). Flow 434 curves revealed a lower viscosity for SIG-APP compared to extracted pectin solutions (Figure 5) 435 while the frequency sweep tests at 25°C showed an opposite trend of G' and G'' (Figure 6A) 436 having a SOL state (G' < G'') for extracted pectin solutions and a GEL state for SIG-APP one (G' 437 >G''). Strain sweep tests allowed to identify the linear viscoelastic region (LVE) which indicates 438 the range in which the test can be carried out without destroying the structure of the sample. LVE is 439 visible in all the extracted pectin (except for CA 40-50) reaching a yield point for strain up to 50% 440 (Figure 6B). On the other hand, the SIG-APP solution shows a narrow LVE with a yield point at 441 5% strain. Furthermore, rheological measurements highlighted the effect of extraction process on 442 the mechanical behaviour of pectin solutions. Indeed, the use of AA or CA strongly influenced the 443 properties of the final solutions, higher viscosity was obtained when the extraction process was 444 performed using AA at 80°C (AA80-25 and AA80-50) while for CA a reduction of the viscosity 445 was observed increasing the temperature and the time (Figure 5). All the tested conditions 446 maintained a SOL state at 25°C however differences in the frequency and strain sweep test plots 447 were observed ascribed to the acidic conditions (CA or AA) used within the extraction process 448 (Figure 6). When CA was used, G' and G'' decreased for the higher temperature while the longer 449 time reduced the stability of the solutions to strain exhibiting a lower yield point. On the contrary, 450 for AA the process at 80°C guaranteed higher G' and G'' values compared to 40°C, however the 451

- 452 extraction time did not affect the mechanical properties of the solutions tested, indeed only a slight
- reduction of G' and G'' values was observed for AA40-50 compared to AA40-25.
- 454 The tested pectin solutions show G' and G'' values of few Pa, highlighting the potential of this
- 455 material to be applied in the field of soft tissue engineering and regenerative medicine as the
- 456 mechanical properties of several human tissues are in the range from few Pa to kPa [50].



Figure 5. Flow-curves of the extracted pectin solutions from different acidic conditions. Apple
 pectin from Sigma-Aldrich (SIG-APP) has been used as control.



460

Figure 6. Rheological properties of pectin solutions obtained from (A) frequency and (B) strain
sweep tests after 25 (left) and 50 (right) mins of extraction. Apple pectin from Sigma-Aldrich (SIGAPP) has been used as control.

465 **3.3** *In vitro* cell tests

466 Neonatal Normal Human Dermal Fibroblasts were seeded on the tissue culture plates with different

- 467 concentrations of the extracted and commercial pectin to assess their cytocompatibility for
- biomedical applications, particularly for tissue engineering and regenerative medicine.
- 469 The NHDF metabolic activity was assessed by using Presto Blue assay (Figure 7) after 48 hours,
- 470 showing a significant increase when the concentration of the dissolved pectin is below 250 μg/mL,
- 471 confirming the results observed by the live/dead staining assay (Figure 8). Interestingly, the AA80-
- 472 25 and CA80-50 exhibited the highest metabolic activity of the NHDF when compared to the

473	remaining sample AA80-50 (at 1000 μ g/mL p < 0.01). However, all the samples containing the
474	extracted pectin encouraged the growth and a quicker spreading of the cells. In contrast, a
475	significant viability reduction was observed on the cells seeded with the commercial pectin. After
476	48 hours, a reduction of more than 50% compared to the other samples was detected at
477	concentrations in the range of 10-50 μ g/mL. Furthermore, the viability of the NHDF was assessed
478	by live/dead staining assay after 48 h of seeding, as shown in Figure 8. Lower concentrations
479	showed a high cell viability and ability to promote cell attachment. NHDF showed the typical
480	elongated and flattened morphology on all the extracted pectin samples and spreading
481	homogeneously along the TCP surface. On the other hand, highest concentrations seemed to have
482	affected the cell behavior. Particularly, from the concentration of 500 μ g/mL, it was noticed
483	different dead cells (labelled in red) mainly for the samples AA80-50 and SIG-APP.
484	Immunostaining assays confirmed the previous results with the cell maintained spindle-shape in the
485	presence of low concentrations of the extracted pectin, while cells at higher concentrations
486	evidenced a rounded shape and cellular contraction with smaller nucleus (Figure 9). This can be
487	related with the cytotoxic effect of pectin confirmed by low metabolic activity detected by Presto
488	Blue assay and Live/Dead staining.



Figure 7. Metabolic activity of Neo-dermal fibroblast cells after 48 hours of seeding in presence of different concentration (from 1000 to 10 μ g/mL) of the extracted pectin. Apple pectin from Sigma-Aldrich (SIG-APP) has been used as control. The results are shown as average ± SD after normalisation to the control of cells seeded on TCPs.



Figure 8. Live/dead images of of Neo-dermal fibroblast cells after 24 hours of seeding in presence of different concentration (from 1000 to $10 \,\mu\text{g/mL}$) of the extracted pectin. Commercial apple pectin purchased from Sigma-Aldrich (SIG-APP) has been used as control. Scale bar= 300 μ m.



Figure 9. Immuno-staining images of of Neo-dermal fibroblast cells after 48 hours of seeding in presence of different concentration (from 1000 to 10 μ g/mL) of the extracted pectin. Commercial apple pectin purchased from Sigma-Aldrich (SIG-APP) has been used as control. Scale bar= 300 μ m.

4. Conclusion

A comprehensive comparison between different processing factors of a combined organic acidic and ultrasound-assisted extraction applied to obtain pectin from apple biowaste was made to evaluate the procedure performance, including yield and physico-chemical properties, to propose an alternative methodology to the mineral acidic extraction. We found in this work that temperature and time mainly influenced the properties of the extracted pectin in terms of extraction yield, GalA content and methoxylation degree, where temperature presented the highest influence on the process. Moreover, we observed that the acid type only showed effect on the ζ -potential of the extracted materials. Considering the highest cytocompatibility of the extracted pectin compared with the commercial one, the evaluated procedure allows to obtain materials that can be proposed for different biomedical applications, including as hydrogels for soft tissue engineering and regenerative medicine, thanks to the low moduli measured through rheology, and as polyelectrolyte for the development of multilayered coating to modify the surface of medical devices and/or to allow the controlled release of biological molecules and drugs.

Conflicts of interest

The authors that they have no conflict of interest to declare for this publication.

Acknowledgement

This work was supported from the ONE Planet studentship that funded the experimental activities

of Michelle Pazmino at Newcastle and Northumbria Universities.

References

- 1. Barreira, J.C., A.A. Arraibi, and I.C. Ferreira, *Bioactive and functional compounds in apple pomace from juice and cider manufacturing: Potential use in dermal formulations.* Trends in Food Science & Technology, 2019. **90**: p. 76-87.
- 2. Service, U.F.A., *Fresh apples, grapes, and pears: World markets and trade*. Washington, DC, 2021.
- 3. Lyu, F., et al., *Apple pomace as a functional and healthy ingredient in food products: A Review.* Processes, 2020. **8**(3): p. 319.
- 4. Zhang, F., et al., *Apple pomace as a potential valuable resource for full-components utilization: A review.* Journal of Cleaner Production, 2021. **329**: p. 129676.
- 5. Eivazzadeh-Keihan, R., et al., *Pectin-cellulose hydrogel, silk fibroin and magnesium hydroxide nanoparticles hybrid nanocomposites for biomedical applications.* International Journal of Biological Macromolecules, 2021. **192**: p. 7-15.
- 6. Syan, V., et al., *An overview on the types, applications and health implications of fat replacers.* Journal of Food Science and Technology, 2022: p. 1-12.
- 7. Munarin, F., M.C. Tanzi, and P. Petrini, *Advances in biomedical applications of pectin gels.* International journal of biological macromolecules, 2012. **51**(4): p. 681-689.
- 8. Barrios-Rodríguez, Y.F., et al., *Cocoa Pod Husk: A High-Pectin Source with Applications in the Food and Biomedical Fields.* ChemBioEng Reviews, 2022. **9**(5): p. 462-474.
- 9. Chandel, V., et al., *Current Advancements in Pectin: Extraction, Properties and Multifunctional Applications.* Foods, 2022. **11**(17): p. 2683.
- 10. Dranca, F. and M. Oroian, *Optimization of pectin enzymatic extraction from malus domestica 'fălticeni'apple pomace with Celluclast 1.5 L.* Molecules, 2019. **24**(11): p. 2158.
- 11. Chen, M. and M. Lahaye, *Natural deep eutectic solvents pretreatment as an aid for pectin extraction from apple pomace.* Food Hydrocolloids, 2021. **115**: p. 106601.
- 12. Cho, E.-H., et al., *Green process development for apple-peel pectin production by organic acid extraction.* Carbohydrate polymers, 2019. **204**: p. 97-103.
- 13. Luo, J., Y. Ma, and Y. Xu, *Valorization of apple pomace using a two-step slightly acidic processing strategy*. Renewable Energy, 2020. **152**: p. 793-798.
- 14. Chen, M., X. Falourd, and M. Lahaye, *Sequential natural deep eutectic solvent pretreatments of apple pomace: A novel way to promote water extraction of pectin and to tailor its main structural domains.* Carbohydrate Polymers, 2021. **266**: p. 118113.
- 15. Zheng, J., et al., *Radio frequency assisted extraction of pectin from apple pomace: Process optimization and comparison with microwave and conventional methods.* Food Hydrocolloids, 2021. **121**.
- Roselló-Soto, E., et al., Application of Non-conventional Extraction Methods: Toward a Sustainable and Green Production of Valuable Compounds from Mushrooms. Food Engineering Reviews, 2016.
 8(2): p. 214-234.
- 17. Priyadarshini, A., et al., *Emerging food processing technologies and factors impacting their industrial adoption*. Critical Reviews in Food Science and Nutrition, 2019. **59**(19): p. 3082-3101.
- 18. Dranca, F. and M. Oroian *Ultrasound-Assisted Extraction of Pectin from Malus domestica 'Fălticeni' Apple Pomace*. Processes, 2019. **7**, DOI: 10.3390/pr7080488.
- 19. Grassino, A.N., et al., *Ultrasound assisted extraction and characterization of pectin from tomato waste.* Food Chemistry, 2016. **198**: p. 93-100.
- 20. Bai, J.-W., et al., *Polyphenol oxidase inactivation and vitamin C degradation kinetics of Fuji apple quarters by high humidity air impingement blanching*. International Journal of Food Science & Technology, 2013. **48**(6): p. 1135-1141.
- 21. Wang, M., et al., *Characterization and functional properties of mango peel pectin extracted by ultrasound assisted citric acid.* International Journal of Biological Macromolecules, 2016. **91**: p. 794-803.
- 22. Chen, J., et al., *Extraction temperature is a decisive factor for the properties of pectin.* Food Hydrocolloids, 2021. **112**: p. 106160.

- 23. Zheng, J., et al., *Radio frequency assisted extraction of pectin from apple pomace: Process optimization and comparison with microwave and conventional methods.* Food Hydrocolloids, 2021. **121**: p. 107031.
- 24. Luo, J., Y. Xu, and Y. Fan, *Upgrading Pectin Production from Apple Pomace by Acetic Acid Extraction*. Appl Biochem Biotechnol, 2019. **187**(4): p. 1300-1311.
- 25. Gharibzahedi, S.M.T., B. Smith, and Y. Guo, *Pectin extraction from common fig skin by different methods: The physicochemical, rheological, functional, and structural evaluations.* International Journal of Biological Macromolecules, 2019. **136**: p. 275-283.
- 26. Barrios-Rodríguez, Y.F., et al., *Infrared spectroscopy coupled with chemometrics in coffee postharvest processes as complement to the sensory analysis.* LWT, 2021. **145**: p. 111304.
- 27. Liew, S.Q., N.L. Chin, and Y.A. Yusof, *Extraction and characterization of pectin from passion fruit peels*. Agriculture and Agricultural Science Procedia, 2014. **2**: p. 231-236.
- 28. Freitas, C., et al., *Extraction of pectin from passion fruit peel*. Food Engineering Reviews, 2020. **12**(4): p. 460-472.
- 29. Broxterman, S.E., P. Picouet, and H.A. Schols, *Acetylated pectins in raw and heat processed carrots*. Carbohydrate polymers, 2017. **177**: p. 58-66.
- 30. Schmidt, U., L. Schütz, and H. Schuchmann, *Interfacial and emulsifying properties of citrus pectin: Interaction of pH, ionic strength and degree of esterification.* Food Hydrocolloids, 2017. **62**: p. 288-298.
- 31. Pawar, R., et al., *Polysaccharides as carriers of bioactive agents for medical applications*, in *Naturalbased polymers for biomedical applications*. 2008, Elsevier. p. 3-53.
- 32. Sancheti, S.V. and P.R. Gogate, *A review of engineering aspects of intensification of chemical synthesis using ultrasound.* Ultrasonics Sonochemistry, 2017. **36**: p. 527-543.
- 33. Rivas, D.F., et al., *Process intensification education contributes to sustainable development goals. Part 1.* Education for Chemical Engineers, 2020. **32**: p. 1-14.
- 34. Patience, N., D. Schieppati, and D. Boffito, *Continuous and pulsed ultrasound pectin extraction from navel orange peels.* Ultrasonics Sonochemistry, 2021. **73**: p. 105480.
- 35. Zhang, L., et al., *Effect of high-intensity ultrasound on the physicochemical properties and nanostructure of citrus pectin.* Journal of the Science of Food and Agriculture, 2013. **93**(8): p. 2028-2036.
- 36. Einhorn-Stoll, U., *Pectin-water interactions in foods–From powder to gel.* Food hydrocolloids, 2018. **78**: p. 109-119.
- 37. Popescu, I., et al., *Double cross-linked pectin beads stable in physiological environment as potential support for biomedical applications.* Journal of Polymer Research, 2021. **28**(11): p. 1-16.
- 38. Mancuso, E., et al., *Potential of manuka honey as a natural polyelectrolyte to develop biomimetic nanostructured meshes with antimicrobial properties.* Frontiers in bioengineering and biotechnology, 2019. **7**: p. 344.
- Ferreira, A.M., et al., *Multilayer nanoscale functionalization to treat disorders and enhance regeneration of bone tissue.* Nanomedicine: Nanotechnology, Biology and Medicine, 2019. 19: p. 22-38.
- 40. Zhang, L., et al., *Influence of rice bran wax coating on the physicochemical properties and pectin nanostructure of cherry tomatoes.* Food and Bioprocess Technology, 2017. **10**(2): p. 349-357.
- 41. Kozioł, A., et al. *Structural Determination of Pectins by Spectroscopy Methods*. Coatings, 2022. **12**, DOI: 10.3390/coatings12040546.
- 42. Kačuráková, M., et al., *FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses*. Carbohydrate Polymers, 2000. **43**(2): p. 195-203.
- 43. Dranca, F., M. Vargas, and M. Oroian, *Physicochemical properties of pectin from Malus domestica 'Fălticeni' apple pomace as affected by non-conventional extraction techniques.* Food Hydrocolloids, 2020. **100**: p. 105383.
- 44. Begum, R., et al., *Structural and functional properties of pectin extracted from jackfruit (Artocarpus heterophyllus) waste: Effects of drying.* International Journal of Food Properties, 2017. **20**(sup1): p. S190-S201.

- 45. Begum, R., et al., *Characterization of Jackfruit (Artocarpus Heterophyllus) Waste Pectin as Influenced by Various Extraction Conditions.* Agriculture and Agricultural Science Procedia, 2014. **2**: p. 244-251.
- 46. Ferreira, D., et al., *Use of FT-IR spectroscopy to follow the effect of processing in cell wall polysaccharide extracts of a sun-dried pear.* Carbohydrate Polymers, 2001. **45**(2): p. 175-182.
- 47. Gómez-Ordóñez, E., A. Jiménez-Escrig, and P. Rupérez, *Molecular weight distribution of* polysaccharides from edible seaweeds by high-performance size-exclusion chromatography (HPSEC). Talanta, 2012. **93**: p. 153-159.
- 48. Zhu, J. and R.E. Marchant, *Design properties of hydrogel tissue-engineering scaffolds*. Expert review of medical devices, 2011. **8**(5): p. 607-626.
- 49. Pancerz, M., et al., *Colligative and hydrodynamic properties of aqueous solutions of pectin from cornelian cherry and commercial apple pectin.* Food Hydrocolloids, 2019. **89**: p. 406-415.
- 50. Władyczyn, A., et al., Novel hybrid composites based on double-decker silsesquioxanes functionalized by methacrylate derivatives and polyvinyl alcohol as potential materials utilized in biomedical applications. Biomaterials Advances, 2023. **146**: p. 213290.